



TITLE:

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CITATION:

Ishiguro, Toshifumi ...[et al]. Absence of DCIR1 reduces the mortality rate of endotoxemic hepatitis in mice. *European Journal of Immunology* 2017, 47(4): 704-712

ISSUE DATE:

2017-04

URL:

<http://hdl.handle.net/2433/230205>

RIGHT:

This is the accepted version of the following article: [Toshifumi Ishiguro, Tetsuya Fukawa, Kotaro Akaki, Koji Nagaoka, Tatsuki Takeda, Yoichiro Iwakura, Kayo Inaba, Kazuhiko Takahara. Absence of DCIR1 reduces the mortality rate of endotoxemic hepatitis in mice.(2017) 47, 4, 704-712], which has been published in final form at <http://dx.doi.org/10.1002/eji.201646814>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving; The full-text file will be made open to the public on 07 April 2018 in accordance with publisher's 'Terms and Conditions for Self-Archiving'; この論文は出版社版ではありません。引用の際には出版社版をご確認ください。 ; This is not the published version. Please cite only the published version.

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Absence of DCIR1 reduces the mortality rate of endotoxemic hepatitis in mice

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Kew words: chemokine, C-type lectin, dendritic cell immunoreceptor (DCIR1), hepatitis,
neutrophil, SHP-2

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Abbreviation: ALT, alanine aminotransferase; Con A, concanavalin A; DCIR, dendritic
cell immunoreceptor; EAE, experimental autoimmune encephalomyelitis; DCs, dendritic
cells; DSS, dextran sulfate sodium; GalN, D-galactosamine; ITIM, immunoreceptor
tyrosine-based inhibitory motif; LPS, lipopolysaccharides; ROS, reactive oxygen
species; SHP, Src homology region 2 domain-containing phosphatase

Abstract

Dendritic cell immunoreceptor (DCIR) is a C-type lectin with an immunoreceptor tyrosine-based inhibitory motif (ITIM). Mice lacking DCIR1 (*Dcir1*^{-/-} mice) show higher susceptibility to chronic arthritis with increasing age, suggesting that DCIR1 is involved in immune modulation *via* its ITIM. However, the role of DCIR1 in acute immune responses is not clear. In this study, we explored its role in acute experimental hepatitis. Upon injection of D-galactosamine and lipopolysaccharide, *Dcir1*^{-/-} mice showed decreased mortality rates and serum levels of alanine aminotransferase (ALT). In early onset hepatitis, serum levels of TNF- α , which primarily cause inflammation and hepatocyte apoptosis, were significantly lower in *Dcir1*^{-/-} mice than in wild type (WT) mice. In the liver of *Dcir1*^{-/-} mice, influx of neutrophils and other leukocytes decreased. Consistently, the levels of neutrophil-chemoattractant chemokine CXCL1/KC, but not CXCL2/MIP-2, were lower in *Dcir1*^{-/-} mice than in WT mice. However, chemotaxis of *Dcir1*^{-/-} neutrophils to CXCL1/KC appeared normal. Pervanadate treatment induced binding of DCIR1 and Src homology region 2 domain-containing phosphatase (SHP)-2, possibly leading to CXCL1/KC expression. These results suggest that DCIR1 is involved in exacerbation of endotoxemic hepatitis, providing a new therapeutic target for lethal hepatitis.

Introduction

Lectin molecules on the surface of immune cells are involved in antigen and pathogen recognition, resulting in antigen presentation and subsequent clearance [1]. During antigen recognition, lectins also transduce cellular signals that induce various types of immune responses. For example, Dectin-1 is well known to induce reactive oxygen species (ROS) [2] and interleukin (IL)-1 β [3] production. On the other hand, the C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) induces production of immunosuppressive IL-10 in cooperation with toll-like receptors (TLRs) [4].

Dendritic cell immunoreceptor (DCIR) [5, 6], a type II C-type lectin, is homologous to hepatic asialoglycoprotein receptors (ASGPRs)-1 and -2, and macrophage lectins [5]. Human DCIR is expressed in various types of immune cells such as dendritic cells (DCs), macrophages, B cells, and neutrophils, but not NK, CD4 and CD8 T cells [5, 7]. In mice, there are four homologues (DCIR1~4) of human DCIR [7]. DCIR recognizes both endogenous and pathogenic ligands, *e.g.*, mannotriose, sulfo-Lewis^a, Lewis^a, Lewis^b and human immunodeficiency virus type 1 (HIV-1) gp140 glycoproteins [8]. It is suggested that human DCIR and mouse DCIR1 and DCIR2 have an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) that can bind to Src homology region 2 domain-containing phosphatase (SHP)-1 and -2 [9, 10] resulting in immunosuppression. Consistent with such a function, human DCIR downregulates cellular protein

phosphorylation after B cell receptor (BCR) ligation [6], and cytokine production *via* TLR8 [11] and TLR9 [12] signaling. *DCIR* polymorphism has also been involved in susceptibility for systemic lupus erythematosus and primary Sjogren's syndrome [13] and in regulatory T cell induction after intravenous treatment with immunoglobulin to alleviate allergic airways disease [14]. Furthermore, we have reported that *Dcir1*-deficient (*Dcir1*^{-/-}) mice tend to develop autoimmune enthesitis with increased bone/cartilage formation with age, type II collagen-induced experimental arthritis [15, 16], and experimental autoimmune encephalomyelitis (EAE) [17]. However, *Dcir1*^{-/-} mice showed lower susceptibility to dextran sulfate sodium (DSS)-induced colitis [18], and experimental cerebral malaria than wild type (WT) mice did [19]. These results suggest that, in different diseases, DCIR1 can have either immune suppressing or enhancing roles.

In the liver, various types of stresses, such as hepatitis virus infection, chronic alcohol abuse, and metabolic disorders, lead to inflammation and influx of leukocytes, causing tissue damage [20]. In a mouse hepatitis model, administration of D-galactosamine (GalN) and lipopolysaccharide (LPS) causes severe inflammation and lethal organ failure. After injection of GalN and LPS, various types of inflammatory cytokines such as TNF- α and IL-6 are rapidly upregulated. TNF- α activates caspase-3, leading to hepatocyte apoptosis [21]. Subsequently, influx of leukocytes into the liver leads to further liver damage. Among the leukocytes, neutrophils are one of the major

effectors in hepatitis, because an inhibitor for neutrophil's elastase is known to relieve liver injury in rats [22], and neutralizing antibodies (Ab) against the chemokines CXCL1/KC and CXCL2/MIP-2, which are chemoattractant for neutrophils, are known to ameliorate experimental hepatitis [23]. These chemokines are upregulated in early stages of hepatitis and cooperate to exacerbate liver damage. It is also reported that ROS produced by neutrophils directly kill hepatocytes in endotoxemic hepatitis [24].

In previous studies, we have analyzed the roles of DCIR1 in various disease models and age groups, from several days to months [15, 17, 18]. However, the roles of DCIR1 in acute immune response are complex. In the present study, we analyzed the susceptibility of *Dcir1*^{-/-} mice to experimental liver injury induced by GalN and LPS in order to further elucidate this point.

Results

Survival rate and liver damage of *Dcir1*^{-/-} mice in response to lethal hepatitis

We first analyzed the survival rate of mice upon induction of endotoxemic hepatitis using GalN and LPS. The results showed a higher survival rate of *Dcir1*^{-/-} than WT mice (Fig. 1A). The levels of serum alanine aminotransferase (ALT), which indicated hepatocyte death, were significantly reduced in *Dcir1*^{-/-} mice at 5 h after hepatitis induction (Fig. 1B). Macroscopic appearance of the liver also showed less damage in *Dcir1*^{-/-} mice than in WT mice (Fig. 1C). Liver histological analysis showed less destruction of hepatic structure with hemorrhage and formation of vacuoles in *Dcir1*^{-/-} mice than in WT mice (Fig. 1D).

The proinflammatory cytokine TNF- α primarily leads to the activation of caspase-3 and hepatocyte apoptosis directly at the onset of hepatitis caused by GalN and LPS [21]. In *Dcir1*^{-/-} mice, serum levels of TNF- α and other cytokines, such as IL-6, IL-10, and monocyte-attractive chemokine CCL1/MCP-1, were also lower than those in WT mice (Fig. 2), suggesting lower responsiveness to GalN and LPS.

Influx of lymphocytes into the liver of *Dcir1*^{-/-} mice during hepatitis

We next checked the cell numbers and populations of leukocytes in the liver of *Dcir1*^{-/-} mice. At steady state, the numbers of CD4⁺ T cells, CD8⁺ T cells, B cells, neutrophils, and monocytes in the liver of *Dcir1*^{-/-} mice appeared normal (Fig. 3A). Five

hours after hepatitis induction, the number of total leukocytes in the livers of WT mice increased approximately 100 fold from that in the steady state (Fig. 3B, top panel). However, the increase in *Dcir1*^{-/-} mice was significantly less than that in the livers of WT mice. Similarly, the numbers of CD4⁺ T cells, CD8⁺ T cells, B cells, and neutrophils were lower in the livers of *Dcir1*^{-/-} mice than in that of WT mice (Fig. 3B, lower panels), being consistent with the liver damage in *Dcir1*^{-/-} mice.

Serum levels of chemokine CXCL1/KC in *Dcir1*^{-/-} mice during hepatitis

Neutrophils are thought to be major initiators and effector cells in several hepatic injury models using concanavalin A (Con A) [25], GalN and LPS [26], and ischemia/reperfusion [27]. Therefore, we focused on the neutrophil chemokines CXCL1/KC and CXCL2/MIP-2, which are involved in endotoxemic liver injury in mice [23], and we found that the serum levels of CXCL1/KC were significantly lower in *Dcir1*^{-/-} mice than in WT mice (Fig. 4). However, the serum levels CXCL2/MIP-2 were identical in both mice types.

Since neutrophils express DCIR1 [7], it is possible that the lack of DCIR1 affects their chemotactic response to CXCL1/KC. However, *Dcir1*^{-/-} and WT neutrophils showed comparable chemotactic responses to CXCL1/KC (Fig. 5). We previously reported that responsiveness of *Dcir1*^{-/-} neutrophils to CXCL2/MIP-2 was also normal [18]. This observation suggests that the low levels of CXCL1/KC expression in the liver of *Dcir1*^{-/-}

mice result in decreased neutrophil influx into the liver and ameliorate liver damage. Taken together, our results suggest that DCIR1 upregulates CXCL1/KC production and subsequent influx of neutrophils, resulting in high mortality rate in endotoxemic hepatitis.

Interaction of DCIR1 and SHP-2

Richard *et.al.* reported that a phosphorylated synthetic peptide containing human DCIR ITIM could bind SHP-2 [10]. It was recently reported that SHP-2 induced human CXCL8/IL-8, a homolog of mouse CXCL1/KC and CXCL2/MIP-2, through p38 MAPK in astrocytes [28], and, in mice, the p38 MAPK inhibitor (SB 239063) suppresses induction of CXCL1/KC and CXCL2/MIP-2 during endotoxemic liver injury *in vivo* [29]. These results suggest that the DCIR1-SHP-2 pathway controls CXCL1/KC expression. However, binding of DCIR1 and SHP-2 has not been shown. Using HEK293T expressing tagged-DCIR1, we found that pervanadate treatment or antibody cross-linking led to DCIR1 phosphorylation (Fig. 6A and B). Such phosphorylation by pervanadate treatment was not observed with C-type lectin SIGNR3 containing hemi-immunoreceptor tyrosine-based activation motif (hemITAM) [30]. Furthermore, pervanadate treatment of HEK293T expressing tagged-DCIR1 induced binding of DCIR1 and SHP-2 (Fig. 6C). These results suggest that ligand-bound DCIR1 interacts with SHP-2 and subsequently enhances CXCL1/KC production.

Discussion

Recently, studies aim at exploring new roles of C-type lectins in addition to their known functions in immune responses to pathogens. In this study, we found that DCIR1 increased TNF- α production at the onset of endotoxemic hepatitis, and subsequent neutrophil influx to the liver, leading to exacerbation of liver damage and lethality.

One of the mechanisms by which neutrophils induce hepatocyte death involves ROS [24, 31]. Batra *et.al.* reported that CXCL1/KC was essential for NADPH oxidase-mediated ROS production of neutrophils in polymicrobial sepsis [32]. We have also reported that lack of DCIR1 in neutrophils decreased ROS production in response to LPS *in vitro* [18]. Therefore, DCIR1 possibly controls not only the influx of neutrophils, but also their cytotoxicity *via* ROS production in the liver. Furthermore, CXCL1/KC is involved in neutrophil migration, cytokine production, NF- κ B and MAPKs activation, and ICAM-1 expression [32]. Therefore, low induction of CXCL1/KC in *Dcir1*^{-/-} mice may be involved in hepatitis amelioration at various steps of the immune response.

We have studied the roles of DCIR1 during aging and in several disease models. Among these, DCIR1 is involved in the suppression of autoimmune enthesitis with increasing age [15, 16] and EAE [17]. However, in DSS-induced colitis [18] and endotoxemic hepatitis (present study), DCIR1 exacerbated the symptoms. In the autoimmune enthesitis and EAE models, the main effector cells are T cells [16, 33], suggesting the intervention of antigen specific immune responses. We have reported that

lack of DCIR1 enhanced GM-CSF signaling, resulting in an increase of DCs [15] that effectively induced antigen specific IFN- γ -producing T cells [16]. On the other hand, acute organ destruction by neutrophils occurs in DSS-induced colitis and in the endotoxemic hepatitis model. Downregulation of CXCL chemokines in *Dcir1*^{-/-} mice suppresses neutrophil influx into the organs and cytotoxicity *via* ROS production. Taken together, these results suggest that DCIR1 enhances the influx of leukocytes and inflammation by upregulating chemokine production in an acute immune response, as well as suppresses excess inflammation by reducing T cell effector function *via* control of the number of DCs in a long-term immune response, resulting in dual pro- and anti-inflammatory roles. Therefore, the discrepancy between immune suppressive and enhancing functions of DCIR1 in the different disease models may be related to the different stages of immune response in each model.

We have reported that, in the DSS-induced colitis model, expression of CXCL2/MIP-2 in the large intestine was downregulated in *Dcir1*^{-/-} mice, leading to neutrophil influx decrease [18]. In M ϕ , expression of CXCL1/KC and CXCL2/MIP-2 were differently controlled by the Myd88-NF- κ B and combined Myd88-NF- κ B and TRIF-IRF pathways, respectively [34]. *Cxcl1/Kc* contains three NF- κ B binding sites in its promoter region, whereas *Cxcl2/Mip-2* contains a typical IRF and two NF- κ B binding sites. Activation of these pathways depends on a combination of TLRs and their ligands. It is therefore possible that in each disease model a different combination of TLRs and

their ligands is involved, explaining the discrepancies we observed with respect to the chemokines affected in *Dcir1*^{-/-} mice. Indeed, in the case of the GalN and LPS hepatitis model, NF-κB activity appears to be controlled by both the TLR4 and DCIR1-SHP-2 pathways. Lack of SHP-2 decreases p65-NF-κB signaling in Mφ [35]. Therefore, NF-κB signaling through DCIR1-SHP-2 pathway may decrease in *Dcir1*^{-/-} mice, leading to low expression of CXCL1/KC in hepatitis. On the other hand, attenuated NF-κB signaling and/or TRIF-IRF signaling may be sufficient for CXCL2/MIP-2 expression, as described previously [34]. The attenuation of NF-κB signaling may be responsible for the low levels of serum TNF-α at the onset of hepatitis.

DCIR1 is expressed in macrophages and DCs [5]. We checked CXCL1/KC production in Kupffer cells and DCs purified from the liver of *Dcir1*^{-/-} mice upon stimulation with LPS *in vitro*. However, the lack of DCIR1 did not decrease CXCL1/KC production under these conditions (Supporting Information Fig. S1). This was also the case in bone marrow-derived DCs. It is possible that the lack of DCIR1 indirectly affects CXCL1/KC production in these cell types *in vivo*. However, DCIR1 may require interactions with certain endogenous ligands [8] to augment CXCL1/KC production in hepatitis, as shown in Fig. 6.

Recently, Greco *et al.* reported that the C-type lectin Mincle exacerbated Con A-induced hepatitis [36]. This result is consistent with previous data showing that Mincle binds with FcRγ adpter molecules at an intracellular immunoreceptor tyrosine-based

activation motif (ITAM), leading to NF- κ B activation and inflammation *via* the Syk-CARD9/BCL10/MALT1 pathway [4]. In this report, mRNA expression of CXCL2/MIP-2 decreased in *Mincle*^{-/-} mice, possibly indicating the involvement of neutrophil chemokines in this hepatitis model. CXCR1/KC, CXCR2/MIP-2, and their human homologue CXCL8/IL-8 are involved in various types of acute liver damage, *e.g.*, alcohol injury, acetaminophen injury, ischemia reperfusion injury after transplantation, and Con A injury [37]. However, in the Con A-induced hepatitis model, which is primarily driven by T cells, the susceptibility of *Dcir1*^{-/-} mice was not significantly different from that of WT mice in a comparison of serum ALT levels (data not shown). Therefore, the DCIR1-CXCL1/KC axis may play different roles in these hepatitis models.

At present, the identity of the cell types susceptible to DCIR1 absence *in vivo*, as well as the signaling pathway from DCIR1 to CXCL1/KC expression are not clear. Nevertheless, our data indicate that DCIR1 with ITIM enhances certain immune responses *in vivo*. DCIR1 is a new therapeutic target for lethal hepatitis. Therefore, the signaling pathway from DCIR1 to CXCL1/KC expression should be examined in future experiments. In mice, DCIR2 is closely related to DCIR1 and contains ITIM [7]. Therefore, DCIR2 may also be involved in the exacerbation of hepatitis.

Materials and Methods

Mice

Dcir1^{+/-} mice were generated using E14.1 embryonic stem cells, and backcrossed to C57BL/6J over 8 times [15]. *Dcir1*^{+/-} mice were maintained under specific pathogen-free conditions. Control WT mice were sibling littermates co-housed with *Dcir1*^{+/-} mice. Male mice were used at 7-9 weeks of age. All experiments were conducted according to our institutional guidelines.

Reagents and antibodies

FITC-conjugated anti-Gr-1 (clone RB6-8C5) was obtained from BioLegend (San Diego, CA). FITC-conjugated anti-CD4 (clone GK1.5) and APC-conjugated anti-CD11b (clone M1/70) were obtained from eBioscience (San Diego, CA). FITC-conjugated anti-B220 (clone RA3-6B2), anti-Ly6G (clone 1A8), PE-conjugated anti-CD3ε (clone 145-2C11), and APC-conjugated anti-CD8 (clone 53-6.7) were obtained from BD Pharmingen (San Diego, CA). Biotin-conjugated F4/80 (clone BM8) was obtained from BioLegend. For removing dead cells in flow cytometry, 7-AAD (7-amino-actinomycin) (Invitrogen, Carlsbad, CA) was used. GalN and LPS (*Escherichia coli* 0111:B4) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant mouse CXCL1/KC and CXCL2/MIP-2 was obtained from R&D Systems (Minneapolis, MN).

Induction of hepatitis

Mice were injected *i.p.* with PBS (200 μ l/20 g mouse weight) containing 100 mg/ml GalN and 40 ng/ml LPS.

Analysis of cytokine production

The levels of MIP-2 were assessed by ELISA using an anti-mouse MIP-2 mAb (clone MAB452) and a biotin-conjugated rabbit polyclonal anti-mouse MIP-2 (R&D Systems) as capturing and detection Abs, respectively. The levels of KC were assessed with the Cytometric Bead Array (CBA) mouse KC flex set (BD Biosciences, Franklin Lakes, NJ). The levels of the rest of the cytokines investigated in this study were assessed with the CBA mouse inflammatory kit and mouse Flex sets (BD Biosciences).

Chemotaxis assay

Responsiveness of neutrophils to CXCL1/KC was evaluated by the Transwell chemotaxis assay. Bone marrow cells (1×10^6 cells) stained with FITC-anti-Ly6G and ACP-anti-CD11b mAb in 100 μ l RPMI 1640 supplemented with 1% BSA and 1% HEPES were placed in a 3- μ m pore size polycarbonate Transwell culture insert (Corning Costar, Tewksbury, MA). CXCL1/KC (10 ng/ml) was added at the bottom chamber. After 1 h in a 5% CO₂ environment at 37 °C, migrating cells were recovered in the bottom compartment and analyzed by flow cytometry.

Analysis of alanine aminotransferase (ALT) in serum

Serum ALT levels were measured using Auto Sera S ALT (Sekisui Medical, Tokyo, Japan) in accordance with the manufacturer's protocol.

Histopathological analysis

Tissues were fixed in Bouin's fluid and embedded in paraffin, and 4- μ m sections were used for histopathological examinations. Sections were stained with hematoxylin-eosin and observed using a BZ-8000 Biozero imaging device (Keyence, Osaka, Japan). Images were processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Cell preparation and stimulation to analyze cytokine production

After systemic perfusion with PBS containing 5 mM EDTA and 100 U/ml heparin (Wako, Osaka, Japan), the liver was digested in 400 U/ml collagenase D (Roche Applied Science, Hague Road, IN) with 5,000 U/ml DNase I (Sigma-Aldrich) for 45 min at 37 °C. For neutrophil fractions, the single cells were suspended in 30% Percoll solution (GE Healthcare Life Sciences, Uppsala, Sweden) and centrifuged at $600 \times g$ for 15 min at 24 °C. The cell pellets were treated with ACK lysis buffer (150 mM NH_4Cl , 1 mM KHCO_3 , and 0.001 mM EDTA) before flow cytometry. For the other leukocytes, single

cell suspensions were prepared in 40% Percoll solution, overlaid onto 70% Percoll solution, and centrifuged at $1300 \times g$ for 10 min at room temperature. Cells at the interface were obtained and subjected to flow cytometry using a FACSCalibur system (BD Biosciences).

For Kupffer cells, single cell suspensions were prepared in 22.5% Percoll solution, overlaid onto 45% Percoll solution, and centrifuged at $1300 \times g$ for 20 min. From the cells at the interphase, Kupffer cells were isolated using biotin-anti-F4/80 mAb and avidin-magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) in accordance with the manufacturer's protocol. For liver DCs, single cell suspensions were prepared in 36% Percoll solution, overlaid onto 54% Percoll solution, and centrifuged at $3000 \times g$ for 20 min. From the cells at the interphase, DCs were isolated using anti-CD11c-magnetic beads (Miltenyi Biotech). Bone marrow-derived DCs (BMDCs) were prepared as previously described [2]. BMDCs were obtained using anti-CD11c-magnetic beads. For cytokine production analysis, cells (2×10^4 cells) in a U bottom well were stimulated with 50 ng/ml LPS for 24 h in RPMI1640 containing 10% FCS, and 50 μ M β -mercaptoethanol.

Construction of expression plasmids

cDNA fragments of the Dcir1 were amplified by PCR using the forward primer 5'-GATCGAATTCATGGCTTCAGAAATCACTTATGCAG-3', the

reverse primer: 5'-GATCGCGGCCGCTAAGTTTATTTTCTTCATCTGACAAAC-3'

and the Phusion High-Fidelity PCR Kit (NEB, County Road, MA), and cloned between the *Eco* RI and *Not* I sites of pEF6/V5-His (Invitrogen), resulting in C-terminal V5-tagged DCIR1. The plasmid for C-terminal V5-tagged SIGNR3 has been described previously [38].

Immunoprecipitation and western blotting

HEK293T cells were maintained in Dulbecco's modified Eagle medium containing 10% FCS and 50 μ M β -mercaptoethanol. The cells were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. The following antibodies were used for immunoprecipitation and western blot analysis: anti-V5 (Invitrogen), anti-phosphotyrosine (PY99) (Santa Cruz, Finnell Street, TX) and anti-SHP-2 (C-18) (Santa Cruz). Transfected cells (1×10^6 cells) were treated with pervanadate (0.03% H_2O_2 , 100 μ M Na_3VO_4), anti-V5 (10 μ g/mL) and Na_3VO_4 , or control mouse IgG and Na_3VO_4 for 10 min at 37 °C. The immunoprecipitation and western blot procedures were performed as previously described [39].

Statistical analysis

Statistical significance was determined using the two-tailed Student's *t*-test.

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Differences in the survival rate of each group were determined by the Wilcoxon test. All experiments were repeated at least two times and representative results are shown.

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Acknowledgements: This work was partly supported by a Grant-in-Aid for Scientific Research (16K08737 to KT) from the Ministry of Education, Japan.

Conflict of interest disclosure: The authors declare no commercial or financial conflict of interest.

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Figure legends

Figure 1. Susceptibility of *Dcir1*^{-/-} and WT mice to acute hepatitis.

(A) Male *Dcir1*^{-/-} (n = 8) and WT (n = 8) mice received *i.v.* GalN and LPS injections. The survival rate of mice was monitored for 24 h. * $p < 0.05$; Wilcoxon test. Data are representative of two independent experiments. (B) Serum ALT levels were analyzed 5 h after GalN and LPS injection (n = 4). Data are representative of two independent experiments. Data are shown as mean \pm SD. ** $p < 0.01$; two-tailed Student's *t*-test. (C) The macroscopic appearance of livers from WT and *Dcir1*^{-/-} mice was compared after 5 h of GalN and LPS injection. (D) Liver sections before (left panels) and 5 h after (middle and right panels) GalN and LPS injection were compared (100 \times magnification). The parts of the middle panels enclosed in rectangles are shown in the right panels at higher magnification (400 \times). (C and D) Representative images from more than three experiments are shown.

Figure 2. Levels of serum cytokines in *Dcir1*^{-/-} and WT mice upon injection of GalN and LPS. Serum cytokines of *Dcir1*^{-/-} and WT mice with hepatitis were analyzed at various time points using CBA (n = 3). Data are shown as mean \pm SD and are representative of two independent experiments. ** $p < 0.01$; two-tailed Student's *t*-test.

Figure 3. Leucocytes in the liver of *Dcir1*^{-/-} and WT mice before and after hepatitis

induction. (A and B) Numbers of total cells and leucocytes in liver of DCIR1^{-/-} and WT mice were analyzed before (A), and after (B) 5 h of hepatitis induction. Bar graphs indicate cell numbers gated in the dot blots. Data are shown as mean \pm SD (n = 3) and are representative of two independent experiments. * p < 0.05, ** p < 0.01 and NS= no significance; two-tailed Student's t -test.

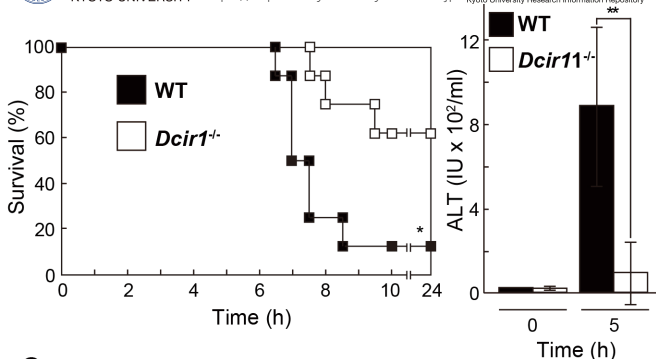
Figure 4. Levels of serum CXCL1/KC and CXCL2/MIP-2 of *Dcir1*^{-/-} and WT mice upon hepatitis induction.

Serum CXCL1/KC (left) and CXCL2/MIP-2 (right) levels were measured after hepatitis induction using CBA and ELISA, respectively. Data are shown as mean \pm SD (n = 6) and are representative of two independent experiments. * p < 0.05; two-tailed Student's t -test.

Figure 5. Responsiveness of neutrophils of *Dcir1*^{-/-} and WT mice to CXCL1/KC. (A and B) After staining with FITC-anti-Ly6G and ACP-anti-CD11b mAb, bone marrow cells were subjected to chemotaxis analyses in the presence of CXCL1/KC using a Transwell assay. After 1 h, cells that had migrated into the bottom well were analyzed by (A) flow cytometry and (B) the rate of neutrophil migration was calculated. Data are shown as mean \pm SD (n = 3). Representative results from two independent experiments are shown.

Figure 6. Phosphorylation of DCIR1 and interaction with SHP-2.

(A) HEK293T cells transfected with a plasmid encoding V5-tagged DCIR1, -SIGNR3 or a control vector, were treated with pervanadate (H_2O_2 and Na_3VO_4), and tyrosine phosphorylation was analyzed by anti-phosphotyrosine Ab after immunoprecipitation with an anti-V5 antibody (upper panel). After stripping, the blot was re-probed with anti-V5 mAb (lower panel). (B) HEK293T cells expressing V5-tagged DCIR1 were cross-linked with a control or an anti-V5 mAb in the presence of Na_3VO_4 . After immunoprecipitation with an anti-V5 antibody, phosphorylation of DCIR1 was analyzed using an anti-phosphotyrosine Ab (upper panel). After stripping, the blot was re-probed with anti-V5 mAb (lower panel). (C) HEK293T cells transfected with DCIR1 were treated with pervanadate as described in (A) and were subjected to immunoprecipitation with an anti-SHP2 Ab. Binding of SHP-2 to DCIR1 was detected using an anti-V5 antibody (upper panel). After stripping, the blot was re-probed with anti-SHP-2 mAb (lower panel). (A-C) Blots are representative of two independent experiments.



C

0 h

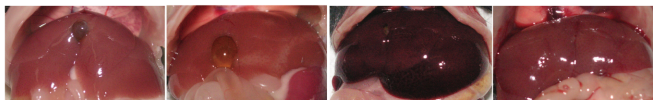
5 h

WT

Dcir1^{-/-}

WT

Dcir1^{-/-}



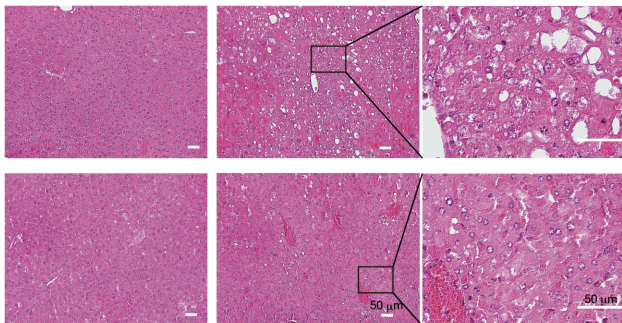
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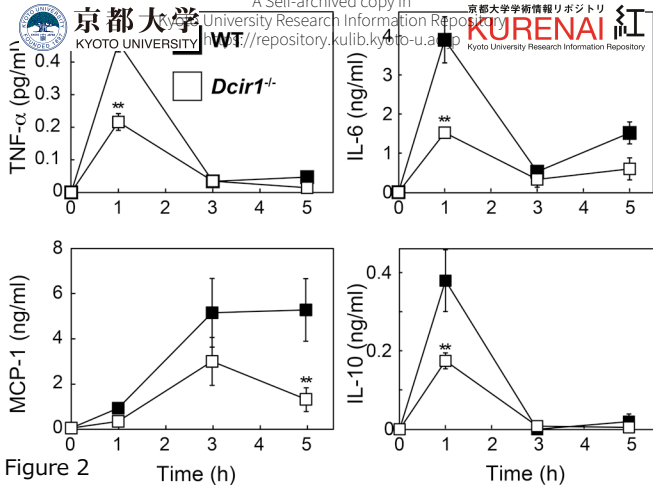
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5 h

WT

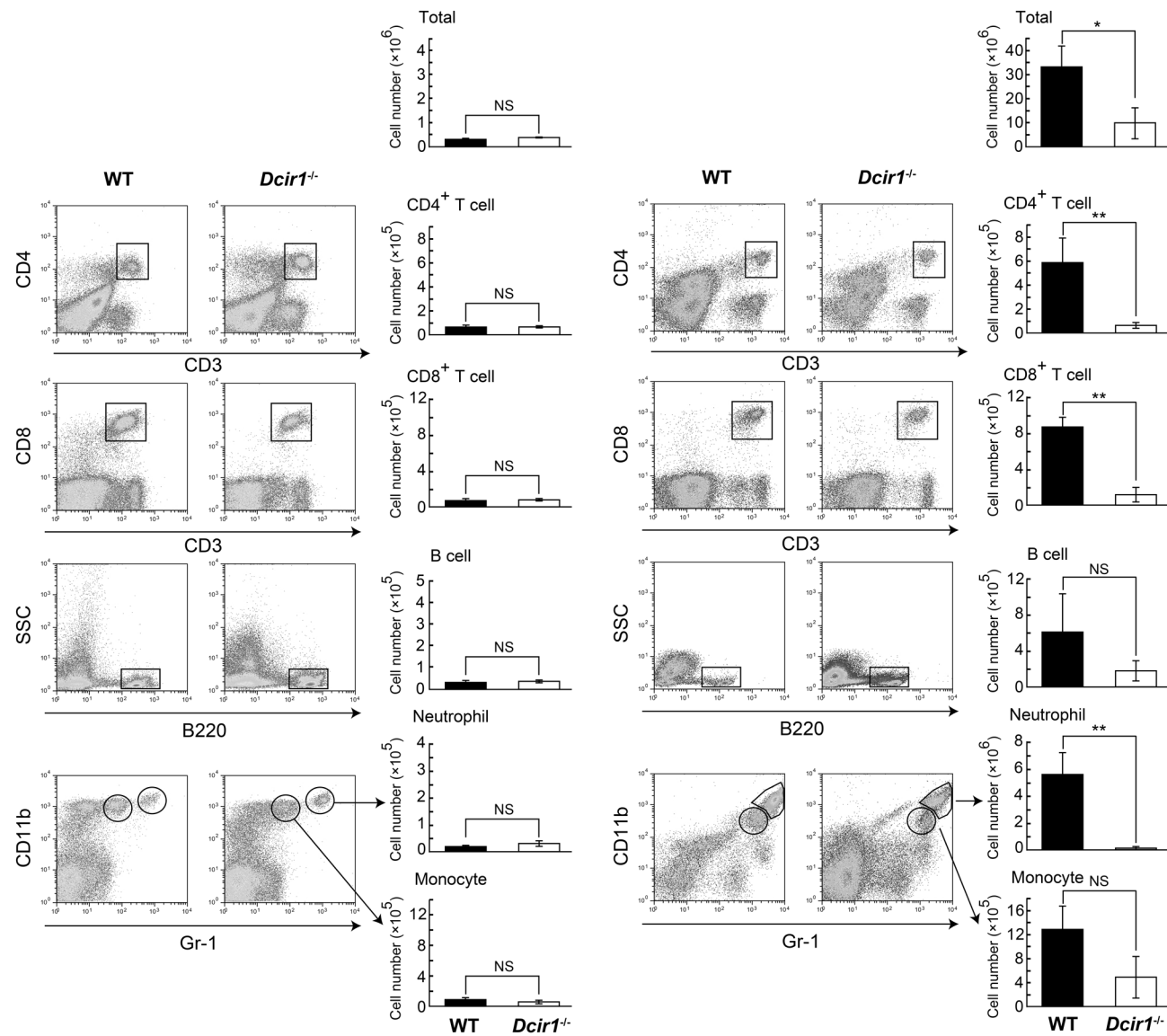
Dcir1^{-/-}

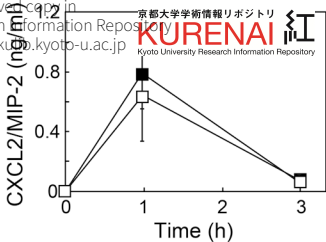
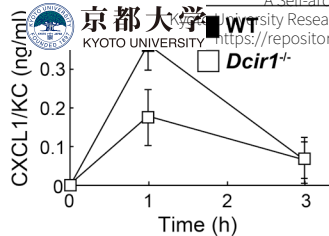




0 h

5 h





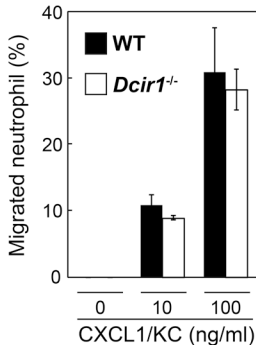
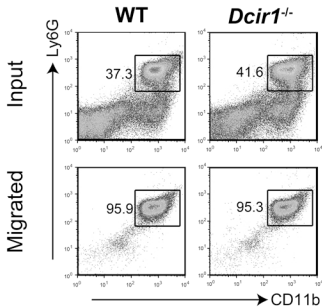
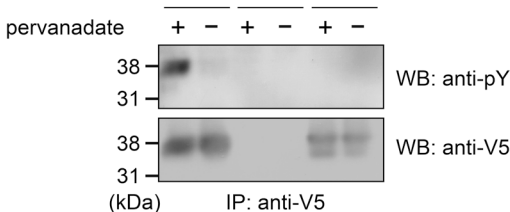
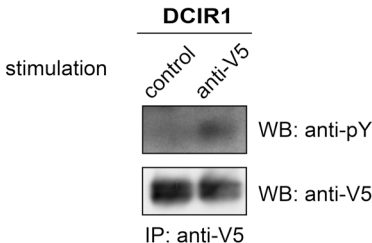


Figure 4



B



C

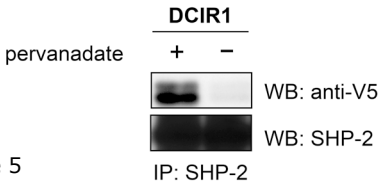


Figure 5